Investigation into the Tumor-Suppressive Effects of the *IFN-ε* Gene on Glioblastoma Proliferation and Viability

Gerald H. Kang

Received September 11, 2024
Accepted December 26, 2024
Electronic access December 31, 2024

Glioblastoma is known to be the most frequent primary brain cancer tumor and also the most aggressive primary brain tumor in adults. The Interferon epsilon (*IFN-ε*) gene was identified after a series of analysis using the cBioPortal database, suggesting its potential role as a novel tumor suppressor gene in patients with brain cancer. This study investigates the effect of the *IFN-ε* gene overexpression on glioblastoma cell proliferation and viability. Glioblastoma cells were transfected with the IFN-ε gene, and results were analyzed across multiple trials. The findings revealed a significant reduction in cell confluency, live cell count, and cell viability in the *IFN-ε* overexpression group compared to the negative control. Specifically, cell confluency was reduced by 20.64%, live cell count decreased by almost half, from 9.12×10^4 cells/mL to 4.72×10^4 cells/mL, and cell viability decreased from 99.9% in the negative control group to 63.4% in the *IFN-ε* overexpression group. Statistical analysis confirmed the significance of these results, suggesting a potential tumor-suppressive role for *IFN-ε* in glioblastoma. This study provides a foundation for further exploration of *IFN-ε* as a therapeutic target in glioblastoma patient treatment.

Keywords: Brain cancer; cBioPortal database; *IFN-ε* gene; Glioblastoma; Gene Therapy

Introduction

Brain cancer is the abnormal growth of cells in or around the brain. Regardless of its status as a benign or malignant tumor, brain cancer poses a significant threat to the patient's life due to the changes and pressures exerted by it on the vital structures of the brain. This fatal nature of brain cancer leads to its high morbidity and mortality rate, making it a crucial global health issue to address.

A previous study indicated that brain and central nervous system cancer have a significant impact on the global burden of disease, ranking 19th among the most frequent malignancies (1.9% of all cancers) and 12th among the leading causes of cancer deaths (2.5% of all cancers)[1]. It shows the consistent rise in the number of new cases and deaths due to brain cancer, emphasizing the escalating public health concern of brain cancer.

Glioblastoma is a type of glioma that originates explicitly from astrocytes, which are glial cells that comprise around 50% of all brain cells. Therefore, they will likely establish direct contact with glioblastoma cells, promoting invasion in the healthy tissues[2]. Glioblastoma is known to be the most frequent primary brain cancer tumor in adults. Yet, it is also the most aggressive primary brain tumor in adults, resulting in its assignment of grade IV, the highest grade in the World Health Organisation (WHO) classification of brain tumors[3]. The classification of glioblastoma as the most aggressive brain tumor can be observed from its median survival time of approximately 15 months after diagnosis or a five-year survival rate of 10%. The recurrence rate is nearly 90%[4]. Given the findings on cBioPortal and recent studies, the following information about the *IFN-ε* gene was revealed: the indication of the *IFN-ε* gene as being involved or having tumor suppressive functions or activities, the vast majority of its gene alteration being deep deletion, and the evident correlation between *IFN-ε* gene alterations and poorer survival outcomes in brain cancer patients. Thus, the primary objective of this study is to determine the impact of *IFN-ε* gene overexpression on glioblastoma cell proliferation, with the broader goal of understanding whether it could act as a tumor suppressor in glioblastoma patients. Overexpression of the *IFN-ε* gene in glioblastoma cells means that its role, previously unavailable in brain cancer cells due to its deep deletion, could be observed. Suppose the *IFN-ε* gene overexpressed glioblastoma cells show a decreased cell proliferation rate. In that case, it will indicate that the *IFN-ε* gene may have tumor-suppressive functions in brain cancer. This could lead to novel therapeutic approaches targeting the *IFN-ε* gene in treating brain cancer.

Methods

Bioinformatics using cBioPortal Database

cBioPortal database was used to analyze the alteration frequency and impact of the *IFN-ε* gene in brain cancer patients. A dataset

of 23,698 genes from 898 patients across 26 studies was examined, focusing on the top 20 most frequently altered genes. Among these, the *IFN-ε* gene was chosen for further investigation due to its novelty and significant alteration frequency. *IFN-ε* alteration was then analyzed across 20 studies involving 7,390 patients categorized into deep deletions, amplifications, and mutations. Kaplan-Meier survival curve was conducted to compare the survival outcomes of brain cancer patients with and without *IFN-ε* gene alterations. The top 20 genes with the highest alteration frequency were selected from the cBioPortal analysis based on data from 23,698 genes across 898 patients (637 living and 261 deceased) and 26 studies. Genes were ranked by their alteration frequency, including deep deletions, amplifications, and mutations. The ranking was based on absolute frequency percentages across the datasets. The log-rank test was used to assess differences in the Kaplan-Meier survival curves. This non-parametric test compares the survival distributions between groups and is standard for survival analysis.

Cell culture and maintenance

A172 glioblastoma cancer cells were purchased from Korea Cell Line Bank. The A172 cell line is derived from a human glioblastoma multiforme (GBM) tumor, which is the most common and aggressive type of primary brain tumor in adults. Specifically, A172 cells represent astrocytic tumors, as glioblastomas originate from astrocytes, a type of glial cell. The cells were grown in Roswell Park Memorial Institute 1640 (RPMI1640) cell culture media. The cell culture media was supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics of penicillin and streptomycin. The cells were maintained healthy at 37 degrees Celsius in a 5% CO2 incubator.

IFN-ε transfection on A172 cell line

IFN-ε overexpression plasmid (HG22269-UT) was purchased from Sinobio. The *IFN-ε* plasmid was transfected with the Lipofectamine 2000 (Invitrogen). After the cells were transfected for 12 hours, the cell culture media was replaced with the fresh cell culture media.

Cell imaging

The cell images were captured using the Nikon inverted microscope. NIS software was used to capture the digital image from the microscope. The cell images were captured in 40X magnification. Cell images were captured 36 hours after *IFN-ε* transfection. Triplicate samples were captured and analyzed to observe the effect of *IFN-ε* on brain cancer cell confluency.

ImageJ analysis to measure the confluency of cells

The ImageJ program was used to analyze each cell image to quantify cell confluency. After images were converted into 8-bit images, a threshold function indicated the area of the cells. After the threshold was adjusted to indicate only the cell portion of the images, the measurement function was used to analyze the percent area of the cells. Triplicate samples were analyzed for negative control (without the *IFN-ε* gene) and *IFN-ε* overexpression (with the *IFN-ε* gene).

Cell viability analysis using Luna-FL cell counter

Cell viability analysis was performed using the Luna-FL automated cell counter device (Logos Bio). The live cells were stained green, and the dead cells were stained red. The overall viability and live and dead cell numbers were analyzed using this counter device.

RT-qPCR with agarose gel

After the transfection of *IFN-ε*, RNA was extracted using the RNA extraction kit (Bioneer) using the manufacturer's provided protocols. Then, RNA was converted into cDNA using the reverse transcription kit (Enzynomics). The synthesized cDNA was used to amplify the *IFN-ε* and GAPDH using the PCR. The amplified genes were analyzed using the agarose gel. The agarose gel was imaged using the Image analyzer (Biorad).

Results and Discussion

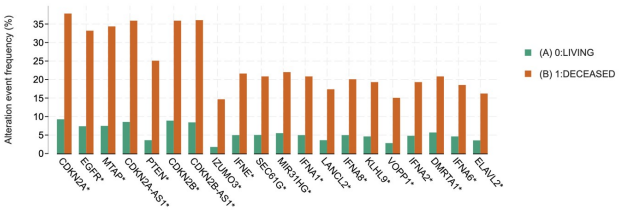

Fig. 1 Bar chart displaying the top 20 genes with the highest alteration frequency in two groups of patients: Living and Deceased. Living subjects are patients with brain tumors that are alive and deceased subjects are patients with brain tumors that have passed away.

The *IFN-ε* gene is a Type-I interferon (IFN-I), a group of pro-inflammatory cytokines produced and recognized by all nucleated cells with the primary aim of blocking pathogens-driven functions[5]. The IFN-I family, including *IFN-ε*, plays a crucial role in modulating immune responses, activating JAK/STAT signaling, and driving typical anti-viral, anti-proliferative, and immunoregulatory responses. As such, they are known to be transient regulators of anti-viral, cell growth, survival, and immune regulatory activities[6]. Acute exposure of cancer cells to

high concentrations of IFN-I has been shown to induce growth arrest and apoptosis. In contrast, chronic exposure to low concentrations offered notable survival advantages to the cancer cells[7].

On the cBioPortal, 23,698 genes were analyzed amongst 898 patients (637 living and 261 deceased) across 26 studies. These genes were then ordered according to their alteration event frequency, with the top 20 most frequently altered genes being displayed on the bar chart. Data showed that the deceased group consistently had higher alteration frequency across all genes presented, suggesting that the alteration of the genes may be linked with the survival outcome of brain cancer patients. Notably, of the top 20 most frequently altered genes, there were 5 IFN-I genes (*IFN-ε*, IFN-A1, IFN-A8, IFN-A2, and IFN-A6, respectively), accounting for 25%. Furthermore, of all the IFN-I genes, it was evident that the *IFN-ε* gene had the highest alteration frequency and the most significant discrepancy in percentage alteration frequency between the living and deceased groups.

Within the human genome, a cluster of thirteen functional IFN genes, including the *IFN-ε* gene, is located at the 9p21.3 cytoband[8]. In 19 different types of cancer, the IFN gene cluster frequently exhibited (7-31%) prevalent homozygous deletions, suggesting that the deletion of IFN-I genes can be associated with the worsening of overall or disease-free survival rates[9]. For instance, the copy number deletion of IFN genes triggers oncogenic pathways while suppressing immune signaling pathways through both the promotion of tumorigenesis and by enabling tumor cells to evade immunosurveillance[10].

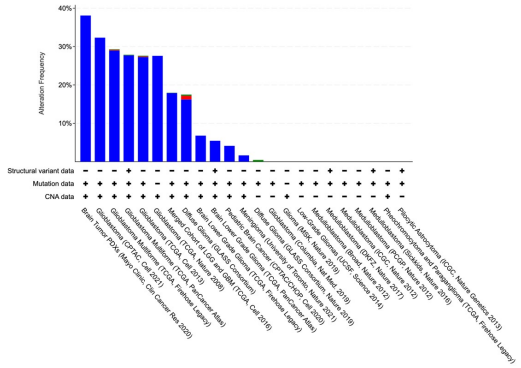

Fig. 2 The bar chart shows the alteration frequency of the *IFN-ε* gene across 20 studies of brain cancer.

In Figure 2, 20 studies, totaling 7,390 patients, were ordered according to the percentage alteration frequency of the *IFN-ε* gene: blue stands for deep deletion, red for amplification, and green for gene mutation. As can be seen, most of the genes altered were due to the deep deletion of the *IFN-ε* gene, thereby confirming that the deletion of the *IFN-ε* gene is the most frequent way in which the *IFN-ε* gene was altered in brain cancer patients.

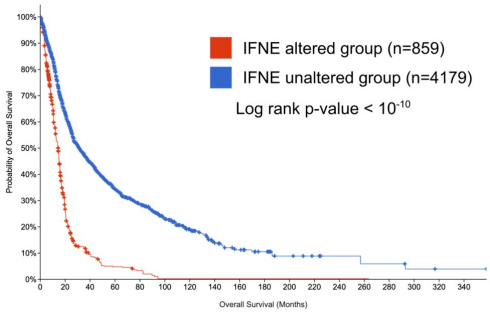

Fig. 3 Kaplan-Meier survival curve comparing the overall probability survival of brain cancer patients dependent on *IFN-ε* gene alteration status.

Figure 3 displays the significant difference in the survival rate of brain cancer patients with or without the *IFN-ε* gene alteration. The *IFN-ε* gene altered group's median survival was 14.49 months, while the unaltered group's median survival was 31.10 months. This demonstrates that the alteration in the *IFN-ε* gene leads to poorer survival outcomes in brain cancer patients, exhibited by a much slower decline in survival probability, nearly half the unaltered group's median. This further strengthens the argument that the absence of the IFN-I genes, specifically the *IFN-ε* gene, could have contributed to the proliferation of brain cancer by removing tumor suppressor genes.

With that being said, the exact function and role of the *IFN-ε* gene is not fully known within the context of brain cancer; it can be inferred from previous research and data that *IFN-ε* may indeed similarly exhibit tumor-suppressive activities. A recent study discovered that the *IFN-ε* gene activated anti-tumor T and natural killer cells while preventing the accumulation and activation of myeloid-derived suppressor cells and regulatory T cells[11]. This meant that the *IFN-ε* gene acted as a tumor suppressor restricting ovarian cancer. As of the time of writing, no research has been done around the role of the *IFN-ε* gene in other cancer types. Hence, this research is novel. Overall, the study's bioinformatics analysis revealed that *IFN-ε* is one of glioblastoma's most frequently deleted genes, which correlates with significantly poorer survival outcomes. This pattern is more pronounced in glioblastoma than in other cancer types, suggesting a unique role for *IFN-ε* in the progression of this aggressive brain tumor.

Figure 4 shows that overexpression of *IFN-ε* in glioblastoma cells significantly increased its mRNA expression level compared to the negative control (p-value = 0.0024). The normalized expression level of *IFN-ε* in the overexpression sample was 1.12, compared to 0.87 in the negative control group. This result shows a 28.74% increase in *IFN-ε* expression. This result indicates that the *IFN-ε* gene transfection system used in this study effectively enhanced *IFN-ε* mRNA expression level in A172 glioblastoma cancer cells. However, the *IFN-ε* protein was not analyzed using the western blot. Therefore, the en-

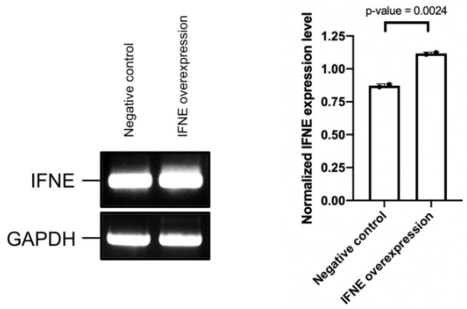

Fig. 4 *IFN-ε* overexpression increased the mRNA *IFN-ε* expression level. The bar chart represents the mean and standard deviation of the normalized FN-*ε* expression level. GAPDH gene expression level was used for the normalization of *IFN-ε* expression level. N=2

hanced mRNA expression in IFNE overexpressed conditions may not affect the protein level.

Figure 5 shows the three trials consisting of eight samples each to evaluate the effect of *IFN-ε* gene overexpression on the proliferation of glioblastoma cells, as indicated by cell confluency. The glioblastoma cells were divided into two groups: a negative control group and an *IFN-ε* overexpression group. In four samples for each trial, the *IFN-ε* gene overexpressed group was transfected with the *IFN-ε* gene. Then, both negative control and *IFN-ε* overexpression groups were cultured and incubated for 36 hours. Cell confluency was subsequently measured using ImageJ software to determine the impact of *IFN-ε* gene overexpression on glioblastoma cell proliferation. The images and data show an evident discrepancy in cell confluency between the two groups. There is a visible decrease in cell confluency between the two groups. This observation is further supported by the results: the negative control group exhibited higher cell confluency across all four samples in all three trials, with an average confluency of 25.14%, while the *IFN-ε* overexpression group had a much more reduced average confluency of 4.50%, resulting in an average difference of 20.64% across the three trials. The results suggest that the overexpression of the *IFN-ε* gene in glioblastoma cells significantly inhibits the ability of the glioblastoma cells to proliferate, marked by a lower cell confluency in the *IFN-ε* overexpression group. This marked difference thus strongly suggests the potential tumor-suppressive role of the *IFN-ε* gene in glioblastoma.

The four samples of the negative control group and the *IFN-ε* overexpression group were combined into one sample each for cell count and viability analysis. Figure 6 illustrates the quantitative impact of the *IFN-ε* gene's overexpression on the glioblastoma cells. The image on the left shows a visual comparison between the negative control and *IFN-ε* overexpression group, with the latter exhibiting a discernible reduction in live cells, as marked by fewer green-highlighted cells (which are the detected live cells by the Luna-FL cell counter). The bar charts on the right show the mean values of the three trials: the live cell count decreased by almost half—the negative control group had a mean of 9.12×10^4 cells/mL. In contrast, the *IFN-ε* overexpression group had 4.72×10^4 cells/mL, and cell viability decreased from 99.9% in the negative control group to 63.4% in the *IFN-ε* overexpression group. The evident discrepancy between the two groups, alongside the statistical significance with the p-value being less than 0.0001, provides strong evidence that overexpression of the *IFN-ε* gene leads to a substantial reduction in both the number of live glioblastoma cells and their viability, further highlighting the profound impact of the *IFN-ε* gene on glioblastoma cell survival.

IFN-ε gene demonstrates a tumor-suppressive role in glioblastoma by inhibiting proliferation and reducing cell viability in the A172 glioblastoma cell line. Mechanistically, *IFN-ε* likely functions through Type-I interferon, which activates the anti-tumor immune response. This aligns with the previous findings that *IFN-ε* overexpression may restrict tumor growth in ovarian cancer by promoting immune surveillance and inducing apoptosis[11].

The physiological role of *IFN-ε* in the context of glioblastoma remains largely unexplored, but the associated functions of Type-I interferon may suggest the potential mechanisms of aggressive brain tumors. Since glioblastoma is characterized by immune evasion and a highly suppressive environment, the frequent deletion of *IFN-ε* in patient samples may contribute to weakened immune surveillance and unchecked tumor growth. Also, previous research indicated that *IFN-ε* induces apoptosis and arrests the growth of cancer cells[12]. This evidence suggests that the deletion of *IFN-ε* could counteract glioblastoma's invasive and proliferative nature.

A limitation of this study is the use of only the A172 glioblastoma cell line without the inclusion of additional glioblastoma cell lines or normal astrocyte controls. While A172 cells are widely recognized and relevant for glioblastoma research, they represent only a single model of this highly heterogeneous tumor type. Including multiple glioblastoma cell lines would provide a broader understanding of the effects of *IFN-ε* overexpression across diverse tumor subtypes, enhancing the generalizability of the findings. Furthermore, the absence of normal astrocyte controls limits the ability to determine whether the observed tumor-suppressive effects of *IFN-ε* are specific to glioblastoma cells or reflect a more generalized cellular response. Additionally, no empty vector control was used, which could have clarified whether the observed effects were solely due to *IFN-ε* overexpression or influenced by the transfection procedure itself. Establishing a dose-response relationship could help determine the threshold and optimal levels of *IFN-ε* required to exert its tumor-suppressive effects and provide insights into whether its impact follows a linear or non-linear pattern. Finally, additional experimental samples would enhance the statistical power of the image analysis. These limitations highlight the need for future studies to incorporate a wider range of glioblastoma models,

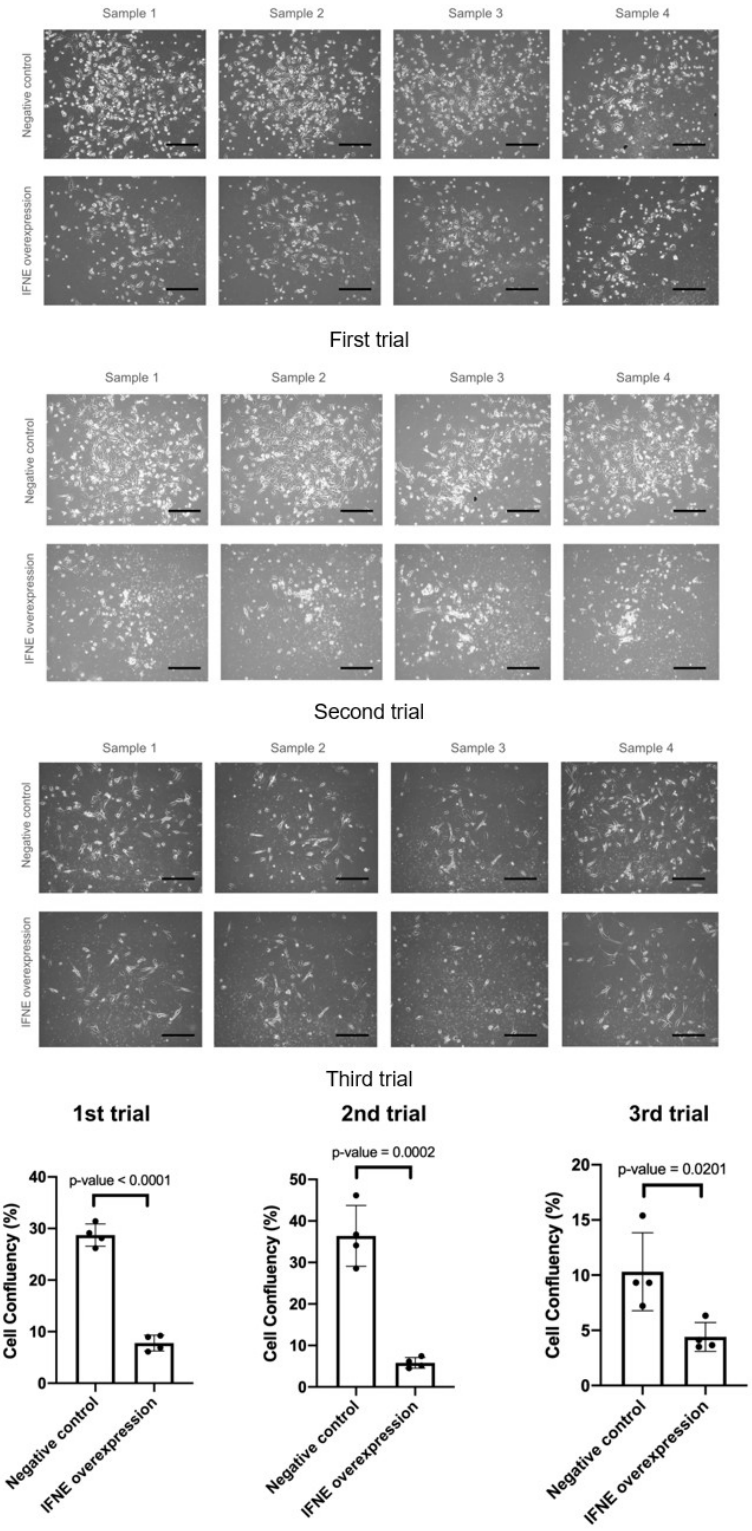

Fig. 5 Microscope images of three trials with four samples per negative control and *IFN-ε* overexpression, with bar charts displaying cell confluency quantified by ImageJ software.

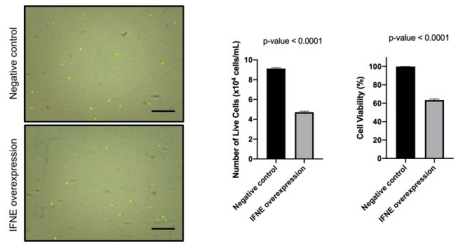

Fig. 6 *IFN-ε* overexpression decreased the number of live cells and viability. The bar chart represents the mean and standard deviation of live cells ($\times 10^4$ cells/mL) and cell viability (%). N=3

normal controls, and robust experimental designs to validate and expand upon the results of this study.

Conclusion

This study provides compelling evidence that the overexpression of the *IFN-ε* gene significantly reduces glioblastoma cell proliferation and viability. The experiments across multiple trials consistently demonstrated a marked decrease in cell confluency, live cell count, and cell viability in the *IFN-ε* overexpression group compared to the negative control group. Specifically, the live cell count in the *IFN-ε* overexpression group was reduced by nearly half, and cell viability dropped from 99.9% to 63.4%, highlighting the impact of the *IFN-ε* gene on glioblastoma cells.

The statistical significance of these results, with consistently low p-values, reinforces the reliability of the findings. Additionally, the increase in mRNA expression of the *IFN-ε* gene following transfection confirms the effectiveness and validity of the transfection system used in this study.

These results suggest that the *IFN-ε* gene plays a crucial role in modulating the behavior of glioblastoma cells, potentially acting as a tumor suppressor. The observed reduction in cell proliferation and viability highlights the importance of further research into *IFN-ε* as a potential therapeutic target for glioblastoma. The findings from this study offer opportunities for future investigations into the mechanisms through which *IFN-ε* exerts its effects and the potential development of *IFN-ε* based therapies for glioblastoma treatment.

Acknowledgements

I want to express my gratitude to Prof. Lee from the University of Suwon for his invaluable support and guidance throughout this project.

Authors

Gerald Kang is a senior at the United World College of South East Asia Dover Campus. He hopes to pursue his passion for biomedical and health sciences through a molecular biology and biochemistry degree, aiming to contribute to advancements in research and innovation in these fields.

References

1 H. Sung, J. Ferlay, R. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal and F. Bray, *Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries*.

2 M. Brandao, T. Simon, G. Critchley and G. Giamas, *Astrocytes, the rising stars of the glioblastoma microenvironment*.

3 H.-G. Wirsching, E. Galanis, M. Weller and G. Handb, *Clin*.

4 J. Park, A. Lomana, D. Marzese, T. Juarez, A. Feroze, P. Hothi, C. Cobbs, A. Patel, S. Kesari, S. Huang and N. Baliga, *A systems approach to brain tumor treatment*.

5 J. Sprooten and A. Garg, *Type I interferons and endoplasmic reticulum stress in health and disease*.

6 L. B. Ivashkiv and L. T. Donlin, *Regulation of type I interferon responses*.

7 H. Cheon, Y. Wang, S. Wightman, M. Jackson and G. Stark, *How cancer cells make and respond to interferon-I*.

8 G. Antonelli, C. Scagnolari, F. Moschella and E. Proietti, *Twenty-five years of type I interferon-based treatment: a critical analysis of its therapeutic use*.

9 A. Razaghi, N. Brusselaers, M. Björnstedt and M. Durand-Dubief, *Copy number alteration of the interferon gene cluster in cancer: Individual patient data meta-analysis prospects to personalized immunotherapy*.

10 Z. Ye, H. Dong, Y. Li, T. Ma, H. Huang, H. Leong, J. Eckel-Passow, J.-P. Kocher, H. Liang and L. Wang, *Prevalent Homozygous Deletions of Type I Interferon and Defensin Genes in Human Cancers Associate with Immunotherapy Resistance*.

11 Z. Marks, N. Campbell, N. Mangan, C. Vandenberg, L. Gearing, A. Matthews, J. Gould, M. Tate, G. Wray-McCann, L. Ying, S. Rosli, N. Brockwell, B. Parker, S. Lim, M. Bilandzic, E. Christie, A. Stephens, E. Geus, M. Wakefield, G.-Y. Ho and P. Hertzog, *Interferon- is a tumour suppressor and restricts ovarian cancer*.

12 M. Abdel-Fattah and H. Saeed, *The Arabian camel, Camelus dromedarius interferon epsilon: Functional expression, in vitro refolding, purification and cytotoxicity on breast cancer cell lines*.